Determining How Changes in Protein Expression Affect Apoptosis in the Jurkat Cell Line

An Honors Thesis

by

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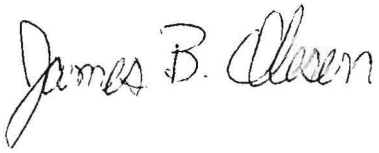

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Abstract

Cancer research is responsible for many of the scientific advances that are evidenced today. Despite the intelligent nature of cancer cells, researchers have still been able to successfully identify and treat the disease effectively. Through the experimentation of Jurkat cells, scientists are able to determine the apoptotic effects of drugs on these cells, whether genes like TAL-1 are inhibited or promoted and also if caspase activity has been inhibited in the cells. By comparing cell counts before and after drug treatments, completing protein extraction and protein determination data, and analyzing Western blot and caspase activity results, concrete conclusions can be derived on the relationship between drug treatment concentrations and apoptosis in Jurkat cells. This data can then be used to introduce drug treatments to human cells for the significant treatment of regularly growing cancer cells.

Acknowledgements

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Author's Statement

In the laboratory setting, Jurkat cells are used to mimic human cells without requiring biohazardous materials or recombinant DNA. To produce data for research, both treated and untreated Jurkat cells were maintained and evaluated. Untreated cells were used as controls to ensure that all flasks contained the correct amount of cells, were viable and comprised the cellular components necessary to conduct experiments. These untreated cells are counted before treatment occurs to ensure data is correct. Treated Jurkat cells start as untreated cells which then have a certain amount of concentrated drug added to them for a twenty-four hour incubation period. After this, cells are once again counted to make sure apoptosis, cell death, has occurred.

Once these preliminary steps have taken place, protein extractions can be done to remove all traces of protein from excess cellular matrix and medium used to grow and feed the cells. In this state, protein can then go through a determination process to compare experimental protein values with known values for protein concentrations in treated Jurkat cells. When these values are proven acceptable, the protein can then be analyzed by a Western blot. This analysis shows separate proteins by size and identifies the different types of protein present within a certain sample. The Western blot will back up our determination of what genes are present and active or present and inactive in the sample. Another assessment is made by using a caspase activity assay. This procedure determines the amount of aspartic-acid specific cysteine proteases present in a sample to ensure that apoptosis is able to take place. In the research, caspase three and eight are used. These caspases are known to be active in aiding apoptosis. Through the data generated, one can identify the magnitude and effectiveness of a drug treatment.

This research has many scientific and medical implications. By finding the correct combination of drug concentration and probing antibodies, the most relevant drug treatments leading to the greatest amount of cell death can be created. The ability of scientists to know the proteins present, which are inhibited or activated by caspase activity, can lead to great advances in the treatment of cancerous cells. If one can understand the genes regulated in cells, it should be much easier to create treatment drugs that can more specifically cause apoptosis to occur.

Introduction

The TAL-1 transcription factor is responsible for growth regulation and differentiation in normally functioning cells, especially in hematopoeisis. When ectopically expressed, TAL-1 produces a cell state leading to the formation of T-cell acute lymphoblastic leukemia (T-ALL) in T-cell development (1). When a mutation occurs in the gene, either a translocation or point mutation, these alterations can lead to a cancerous state because of the aberrant expression of TAL-1 (5). Increased expression of TAL-1 allows for uncontrolled cell growth (through some ill-defined mechanism), which is the case for cancer formation (2). It appears that this transcription factor targets genes that are required to interrupt normal T-cell development. This, in turn, causes the formation of T-ALL (10). Additionally, TAL-1 has been shown to act as a transcriptional repressor and aids in the binding of transcription factor complexes to the E-box sequence (11). After binding to the E-box sequence, TAL-1 can interact with LMO1 or LMO2, which then allows interaction between TAL-1 and GATA-3. GATA-3 binding activates RALDH2 which stimulates the production of retinoic acid. Retinoic acid is responsible for growth and development of the embryo. However, the production of retinoic acid also inhibits cell death which allows the proliferation of cells resulting in T-ALL (12-14). Studies have also shown that ectopic expression of TAL-1 can also be related to the low rates of treatment success of chemotherapy in T-ALL (3). The role of TAL-1 is very important as the transcription factor is found to be aberrantly expressed in 60% of all T-ALL cases (4).

Apoptosis is the method by which cells complete programmed cell death (7). This is a normal, regulated process that the cell enters to maintain proper cellular structure, organization and function. This process is necessary for removing cells that are no longer viable, are incorrectly functioning or interfere with the overall structure of the tissue. Programmed cell

death occurs through the use of caspases. Caspases are important in initiating the pathway of interactions (caspase cascades), leading to apoptosis. Caspases are proteases that contain cysteine residues that have the ability to cleave other proteins (8). There are three different classes of caspases: initiator, effector, and ICE-like proteases. This research dealt exclusively with initiator and effector caspases. Caspase-3 and caspase-8 are usually expressed in cells as pro-caspases and when these caspases cleave themselves or other proteins, they are then able to send pro-apoptotic signals to cause cell death. For example, caspase-8 is an initiator caspase that cleaves caspase-3 to start the process of apoptosis.

This entire process can be completed through receptor-mediated apoptosis or mitochondrial-based apoptosis. In the case of mitochondrial-based apoptosis (an intrinsic pathway), cytochrome c is essential to the overall pathway as it helps activate caspase-9, which in turn, activates caspase-3. Cytochrome c carries electrons through a small channel between mitochondrial membranes which is important for cells in recognizing apoptotic stimuli (16). Due to a change in the membrane potential, pores open within the membrane to allow release of cytochrome c. When apoptosis is initiated, the mitochondrial release of cytochrome c begins caspase activation through binding to apoptosis activating factor 1 (APAF-1). The APAF-1 and cytochrome c complex then joins with pro-caspase-9 to form an apoptosome complex. Through the process of autoproteolysis, activated caspase-9 is released. The release of active caspase-9 will then cleave pro-caspase-3 to the active form of caspase-3 (8). Activated caspase-3 initiates apoptosis through the cleavage of numerous target proteins.

For receptor-mediated apoptosis (an extrinsic pathway), cell death occurs by way of ligands binding to receptors on the plasma membrane of a cell. These receptors are part of the tumor necrosis family of receptors which include TNF-R1, TNF-R2 and Fas-R. The trimeric

receptor then binds adaptor proteins such as Fas associated death domain (FADD) and TNF receptor associated death domain (TRADD). The death domain on the cytoplasmic side of the death receptor will then interact with and bind to the death domain on the adaptor protein. Then, the death effector domain on the adaptor protein associates with the death effector domain on pro-caspase-8. This interaction ultimately forms the death inducing signaling complex (DISC). The DISC complex acts as a recruiter for other pro-caspases to bind to additional adaptors and get them in optimal positions to activate initiator caspases through the process of autoproteolysis. Once cleaved, active caspase-8 cleaves pro-caspase-3 to activate it. Caspase-8 also cleaves BID, a member of the BCL-2 family of proteins, which also signals the release of cytochrome c. Because of apoptotic stimulation, BID moves to the outer mitochondrial membrane and blocks the activity of BCL-2 and BCL-xL (other members of the BCL-2 family). Both BCL-2 and BCL-xL suppress apoptosis, so their inhibition is necessary to allow cell death to occur. Blocking these two members of the BCL-2 family allows Bax and Bak homodimers to create pores within the mitochondrial membrane to release cytochrome c. Stimulation from cytochrome c initiates the cascade of events just listed (intrinsic pathway). Within the BCL-2 family, there are survival proteins such as BCL-2, BCL-xL and Mcl-1 that prevent apoptosis for occurring, while other members such as Bax, BCL-xS, Bak and Bid apoptosis in a cell. To encourage apoptosis, BCL-2 death factors bind to the BCL-2 survival factors. Maintaining the integrity of the outer mitochondrial membrane is an essential function of this family of proteins. Members of this family are found in the cytoplasm or in the membranes of mitochondria, the nucleus and the endoplasmic reticulum (17). In these positions, BCL-2 proteins sense changes within the cell and respond accordingly to external stimuli as either survival or death factors.

To induce apoptosis, staurosporine (STS) and tumor necrosis factor (TNF) are commonly used in varying concentrations as chemotherapeutic drugs. Staurosporine (STS) is a protein kinase C inhibitor that, over a range of concentrations, is known to induce cell apoptosis. Protein kinase c (PKC) is used for many processes in the cell, including cell growth and apoptosis. Studies show that the lack of inhibition of PKC isoenzymes contributes to tumorigenesis due to excessive proliferation of cells without regulated cell death (18). PKC isoenzymes such as PKCα and PKCδ are responsible for initiating apoptosis through their overexpression (18). PKC inhibitors such as STS can stop continued cell cycle progression through inhibition of PKCs. STS has the ability to force cells into cell death (20). As it is a general kinase inhibitor, STS is highly competitive within cells in reaching the kinase binding domain before the cell's ATP. Staurosporine is also able to damage the integrity of membranes, further stimulating the release of cytochrome c and the activation of caspases (20). Tumor necrosis factor (TNF) is a cytokine that is known to aid in the regulation of many cell processes, including apoptosis. TNF works to promote apoptosis by inducing receptor-mediated activation of the initiator caspase-8 and the effector caspase-3 (9). Inhibition or mutation of TNF has been shown to participate in the formation of cancer through the loss of regulated cell death. In this case, TNFα releases cytotoxic elements in the cell. Macrophages within the cellular matrix respond to these cytotoxic elements by producing cytokines. In turn, the cytokines produce apoptotic stimuli to signal the cell to enter cell death pathways. And when TNF remains expressed normally or in increased concentrations within the cell, apoptosis is triggered (18). The lack of TNF expression removes the presence of signaling in the cell, thus causing apoptosis to occur at a lesser rate, if at all. Alteration in the interaction between the receptor and TNF ligand also changes the function of the receptor, also leading to a potentially cancerous state (6).

This research was designed to assess the role of TAL-1 expression on apoptosis. Relationships were assessed through cell counts, protein extraction and determination, Western blotting and caspase activity assays. Through this work, we wanted to determine if the chemotherapeutic drugs used in this research had an effect on the expression of caspase-3 and caspase-8 (both cleaved and uncleaved forms). In the end, the goal of the experimentation was to determine if drug-induced cell death was influenced by the expression of TAL-1 or other proteins involved in some apoptotic pathway.

Materials and Methods

Cell Culture, Cell Counts, and Drug Treatments

Jurkat cells were cultured and grown using RPMI + 10% BGS (bovine growth serum) media in a T-25 flask. These cells were incubated at 37°C in 5% CO_2 and given fresh media every two days. Cell counts were done before each drug treatment using trypan blue exclusion to assess proliferation. The drugs used were staurosporine (STS) and tumor necrosis factor (TNF). STS treatments were made at 0.05 μM and 0.1 μM. TNF treatments were made at 10 mg/μL and 25 mg/μL. One milliliter of each drug was added into an entire flask of cells. Cells were then placed in the incubator for 24 hrs. Following the drug treatment, cells were resuspended to break up clumps of cells. Then, cell counts were done to assess growth and viability.

Protein Extraction

To begin the process of protein extraction, cells were transferred to a 15 mL conical tube and then centrifuged at 1,000 rpm for 5 min to pellet the cells. Media was aspirated and the cells entered a washing stage with 3 mL of cold PBS. The conical tube was centrifuged again at 1,000 rpm for 5 min to pellet cells and the supernatant was poured off. The preceding washing step with cold PBS was then repeated. Cells were once again pelleted and the supernatant poured off again as well. Lysis of cells occurred by adding 1 mL of cold RIPA buffer to a microcentrifuge tube containing either untreated or treated Jurkat cells. The microcentrifuge tube was placed on ice for 45 min before the cells were passed through a 21 gauge needle to aid in the lysis of cells. The cells were then centrifuged for 15 min at 14,000 x g to remove cellular debris. The supernatant was then aliquoted for protein determination and stored at -20°C for subsequent analysis.

Protein Determination

For protein determination, protein samples were first thawed on ice. A number of different combinations of distilled water and BSA protein standard were created. For the first determining step, 1 μL of BSA protein standard was added to 799 μL of distilled water. For the other tube, 5 μL of BSA protein standard was added to 795 μL of distilled water. For each of the protein samples from Jurkat cells, 5 μL of treated cell protein extract was added to 795 μL of distilled water. To all the microcentrifuge tubes, 200 μL of Bio-Rad developing reagent was added. All tubes were then vortexed and assessed for color development.

Following this visual assessment of protein content, a standard curve was generated based on various amounts of BSA protein standard, sterile distilled water and Bio-Rad developing reagent. The amount of protein samples added to each tube varied. Between 1 μL and 5 μL of sample were put into tubes based on the blue color generated during the visual assessment of protein concentration. Based on these values, the amount of sdH_20 varied between 799 and 795 μL to which 200 μL of Bio-Rad developing reagent was added. Absorbance values for all samples were made at 595 nm. The absorbance values generated from the protein standards were then used to produce a standard curve. Microsoft Excel was used to generate the curve and a slope equation such that the protein concentration of each treated sample could be determined.

Western Blotting

Next, Western blotting was performed to identify certain proteins present in the cellular extracts. Based on the presence or absence of proteins and the intensity of individual bands, we were able to assess changes in protein expression. The running gel consisted of a combination of 4 mL sterile distilled milli-Q water, 3.33 mL 30% acrylamide/0.8% bisacrylamide (pH 8.8), 50 μL 10 % ammonium persulfate (APS) and 10 μL TEMED. This solution was pipetted between the glass plates in the apparatus until just below the top of the smaller plate. Then, the running gel was allowed to polymerize for at least 15-20 min.

While polymerization of the running gel occurred, a stacking gel was prepared using 3.35 mL sterile distilled milli-Q water, 650 μL 30% acrylamide/0.8% bisacrylamide (pH 6.8), 1.25 mL 4X Tris/SDS, 50 μL 10% APS and 5 μL TEMED. When the running gel polymerized, the

gel was inverted and Kimwipes were used to remove the excess liquid before adding the stacking gel. A comb was inserted into the stacking gel to form the wells and this was also allowed to polymerize for 15-20 min. During this period, 1000 mL of 1X SDS running buffer was made from a 5X stock solution. After polymerization, the comb was carefully pulled out of the stacking gel and wells were rinsed with running buffer. Small pipette tips were used to aspirate the running buffer from the wells. The standard curve (prepared based on protein determination) was used to calculate how many microliters of sample to add to each well. When this was done, one microliter of sample buffer was added to each tube and then boiled for 5 min. The samples were then loaded into their respective lanes. Running buffer was added and the gel was run at 100V for 1- 1.5 hrs.

When the dye front reached the bottom of the plates, the power supply was turned off and semi-dry transfer was completed. The gel was transferred onto a nitrocellulose membrane at 90 mA per gel for 1 hr. The membrane was then washed with 3 mL of 1X PBS for 5 min, drained and the process was repeated three times. The membrane was placed in 3 mL of Odyssey blocking buffer and rocked on a platform for 1 hr at 4°C. Immediately following, 3 mL of blocking buffer, 3 µL of Tween-20 and 3-6 µL of primary antibody were mixed together. Primary antibodies in the Odyssey blocking buffer solution were added to the respective membranes and allowed to rock on a platform rocker overnight at 4°C.

The following day, the membrane was drained and washed four times with 1X PBS/0.01% Tween-20 for 5 min each wash. Then, 3 µL of Tween-20, 3 mL of Odyssey blocking buffer and 3 µL of goat anti-rabbit secondary antibody was added to each membrane and rocked for 1 hr. After incubating in the secondary antibody, the membranes were washed four times in 1X PBS / 0.01% Tween-20 for 5 min each wash. Then, the membrane was washed with 1X PBS

twice for 5 min each. Membranes were kept in 1X PBS until imaged using the LI-COR Odyssey Imaging System.

Capase Activity

Caspase activity was assessed using a caspase activity kit. A series of samples were set up in duplicate in a 96-well plate that consisted of a blank, negative control and induced apoptosis samples. To each well, 32 µL of caspase assay buffer, 2 µL of DMSO and 10 µL of DTT were added. How many microliters of cell sample was added was dependent upon the concentration of protein in each sample. Then, sterile distilled milli-Q water was added to bring the total volume in each well to 98 µL. The last addition was 2 µL of the DEVD-pNA substrate to each of the wells. After sealing the plate with parafilm, the plate was incubated for 4 hrs. The plate was then analyzed for caspase activity using a Tecan microplate reader. Caspase activity was measured based on cleavage of the DEVD-pNA substrate by caspase-3. All wells were read at 405 nm.

Results

Protein Expression in Cultured Jurkat Cells

Cell Counts

Cell counts were completed to assess cell viability after drug treatment (Table 1). To establish the correct drug concentration to use, cells were cultured and counted twice a week. Treatments were made over a 24 hr period and a trypan blue exclusion cell count was conducted. Various concentrations of staurosporine (STS) and tumor necrosis factor (TNF) were evaluated. All of the treated flasks showed at least minimal amounts of cell death, showing the distinction between untreated and treated cells. Initial treatments indicated a decrease in cells by at least $2.0x10^6$ cells. These values indicate that the higher drug concentrations did cause the most initial cell death. Subsequent treatment data determined STS treated cells showed an increase in cells. Both TNF treatments had the same trend as the first treatment and data showed fewer cells than the untreated sample. Following this treatment, cells decreased in all samples by $2.2x10^6$ cells. Here, the low drug concentration of both the STS and TNF treatments caused greater cell death than the high drug concentration. From the cell count data, both TNF drug concentrations cause the most initial cell death (Table 1).

When compared between the high and low concentrations, STS 0.5 had the lowest amount of living cells (the most cell death) in all cell counts. STS 0.1 had more living cells than STS 0.5 in all the treatment results. As for the TNF treated cells, TNF 25 had the lowest amount of cell death in comparison to the TNF 10 cells. Although the STS and TNF treatments varied in the concentration that produced the highest amount of cell death, the data was consistent and did

not vary from treatment to treatment.

Date	Untreated	STS 0.05	STS 0.1	TNF 10	TNF 25
9/29/2010	$3.05x10^6$	$1.0x10^6$	$1.4x10^5$	$4.25x10^5$	$2.0x10^5$
2/8/2011	$1.23x10^6$	$1.46x10^6$	$1.85x10^6$	$1.15x10^6$	$8.85x10^5$
2/11/2011	$2.74x10^6$	$3.95x10^5$	$4.55x10^5$	$2.71x10^5$	$3.67x10^5$

Table 1. Cell counts of untreated and treated cells. All cells were kept at 37°C and 5% CO_2 for culture and treatment. Various concentrations of staurosporine (STS, 0.05 μM and 0.1 μM) and tumor necrosis factor (TNF, 10 μg/μL and 25 μg/μL) were used.

Western Blotting

Western blotting was then completed to assess protein expression differences between untreated and treated cell extracts. As stated in the Materials and Methods section, the first two membranes were probed with actin and cleaved caspase-3 or cleaved caspase-8. In Figure 1, no difference in the intensity of actin expression was seen across all loaded samples (Figure 1). Since actin is ubiquitously expressed at a high level in all cells, no difference should be detected (dark bands in all lanes). However, the expression of cleaved caspase-8 did vary between samples.

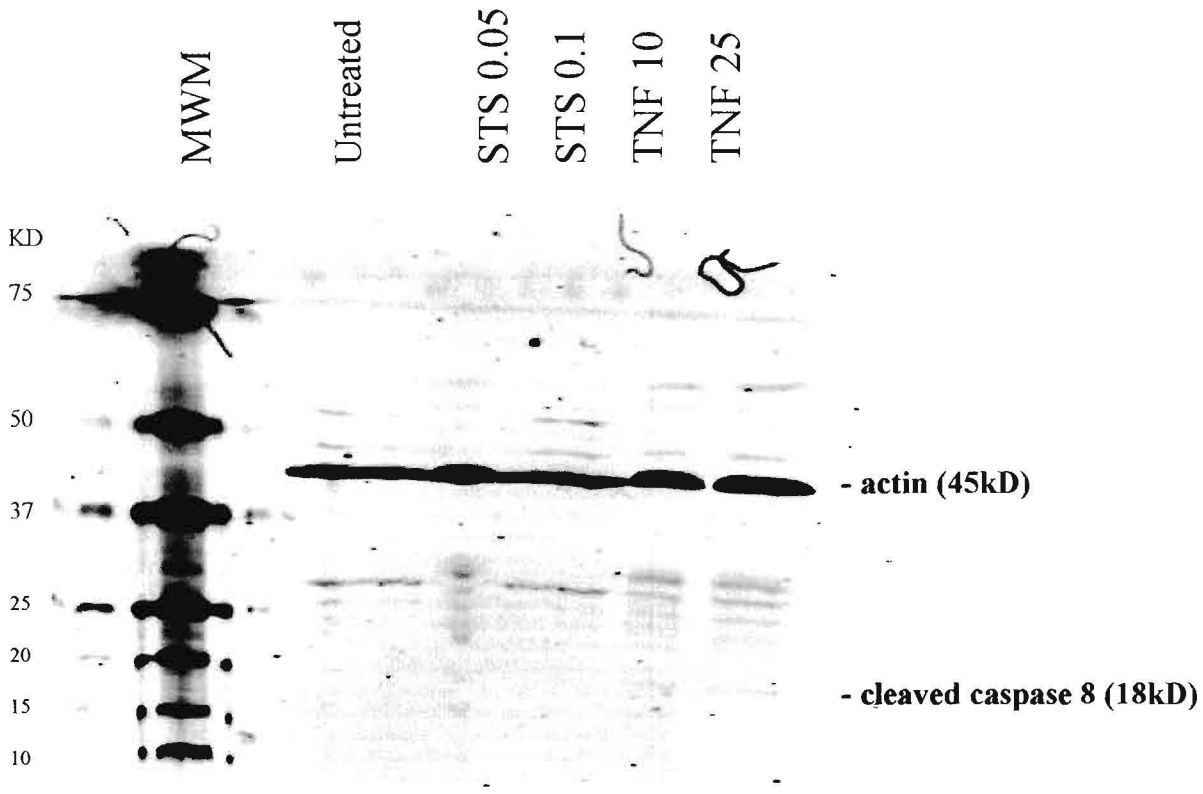

Figure 1. Western blot of Jurkat cell extracts probed with actin and cleaved caspase-8. Jurkat cells were either untreated or treated for 24 hrs with STS (0.05 μM or 0.1 μM) or TNF (10 μg/μL or 25 μg/μL). The Odyssey Imaging System was used to determine band intensity and to correct contrast issues caused by the background.

The STS 0.05, TNF 10 and TNF 25 treated cells showed the greatest intensity of cleaved caspase-8 expression. The other treated extracts did not have faint bands which indicated no expression of cleaved caspase-8 in the untreated and STS 0.1 samples. The expression of cleaved caspase-8 at 41/43 kDa for all treatments was non-existent. No visible band was detected in any lane. The expression of cleaved caspase-8 at 18 kD was somewhat better in terms of visibility, but still was not evident in the untreated and STS 0.1 treated extracts. Extremely faint bands were seen in both TNF-treated extracts. Based on these results, cleaved caspase-8 expression was minimal in the TNF-treated extracts and not detected by the Western blotting in the untreated

and STS 0.1treated extracts. Although it was known that there should have been minimal activity in all cells, this study did not confirm those beliefs. Non-specific antibody interactions also occurred, as the membrane showed faint bands in all lanes (Figure 1).

When blots were treated with actin and cleaved caspase-3 antibodies, the intensity of the actin band was the same in all samples at 45 kD (Figure 2). Again, this was expected as actin is known to be expressed in all cells. In terms of cleaved caspase-3 (17/19 kD) expression, there were no variances observed across all samples. No bands were observed in any lane (no expression). This image implied that cleaved caspase-3 was not active in any sample and did not have any effect on apoptosis in the extracts. Non-specific antibody interactions also occurred, as depicted by faint bands in all samples (Figure 2).

Additionally, a Western blot was probed with both antibodies against uncleaved caspase-3 and caspase-8 (Figure 3). This image showed that these proteins were expressed. Full-length, inactive caspase-3 appeared to be equally expressed (35 kD), but the cleavage fragments at 17/19kD were not (Figure 3). Both TNF-treated samples showed greater intensity of bands than the untreated, STS 0.05 and STS 0.1 samples. This distinction suggests that TNF-treated cells had all forms of caspase-3 readily available, with higher levels of cleaved caspase-3 present. When compared to Figure 2, Figure 3 shows that both caspases were expressed in the greatest amounts when in the uncleaved form. Cleaved caspase-3 was not readily expressed in Figure 2, as shown by the lack of bands at 19 kD. However, Figure 3 displays that the uncleaved and cleaved forms of caspase-3 and caspase-8 have moderate expression in all extracts. In the case of full-length, inactive caspase-8, the untreated and STS treatments showed the greatest amount of expression. The bands at 55 kD in TNF-treated samples were very faint in comparison to STS-treated and untreated lanes. This membrane also had evidence of the cleaved version of caspase-

8 (18 kD). It was hard to detect the bands for caspase-8 at 18 kD in untreated and STS-treated extracts. There were faint bands in the TNF-treated extracts, but intensity differences between the TNF 10 and TNF 25 extracts could not be readily determined. Based on the Western blot, it was found that TNF-treated extracts had the greatest expression of the active form of caspase-8 (Figure 3). When compared, Figures 1 and 2 were both probed with actin while Figure 3 was not. The prominence of the bands in Figure 3 was much more difficult to detect than that of Figures 1 and 2. This may have been caused by unequal loading of each sample. Also, Figure 3 had considerably more non-specific antibody interactions than the other two figures.

Next, expression of BAD and p-BAD was assessed in all samples (Figure 4). As both BAD and p-BAD have similar molecular weights, it was difficult to resolve them as separate bands. Expression was minimal for p-BAD (18/19kD) in all samples present on the membrane. The greatest expression shown was in the extracts from TNF 10 and TNF 25 treatments. As for BAD (23 kD), expression was much more pronounced, especially in the STS 0.05 and TNF 10 treatments. However, there was little or no expression present in the untreated and STS 0.1 treated extracts. Out of the remaining protein samples, the STS 0.05 and TNF 10 treatments had the most intense amount of expression (Figure 4). Of all of the samples, STS 0.05 displayed the most prominent band intensity at 23 kD for BAD followed by TNF 10 and TNF 25. If any BAD expression was present in the untreated and STS 0.1 extracts, it was hard to detect due to background.

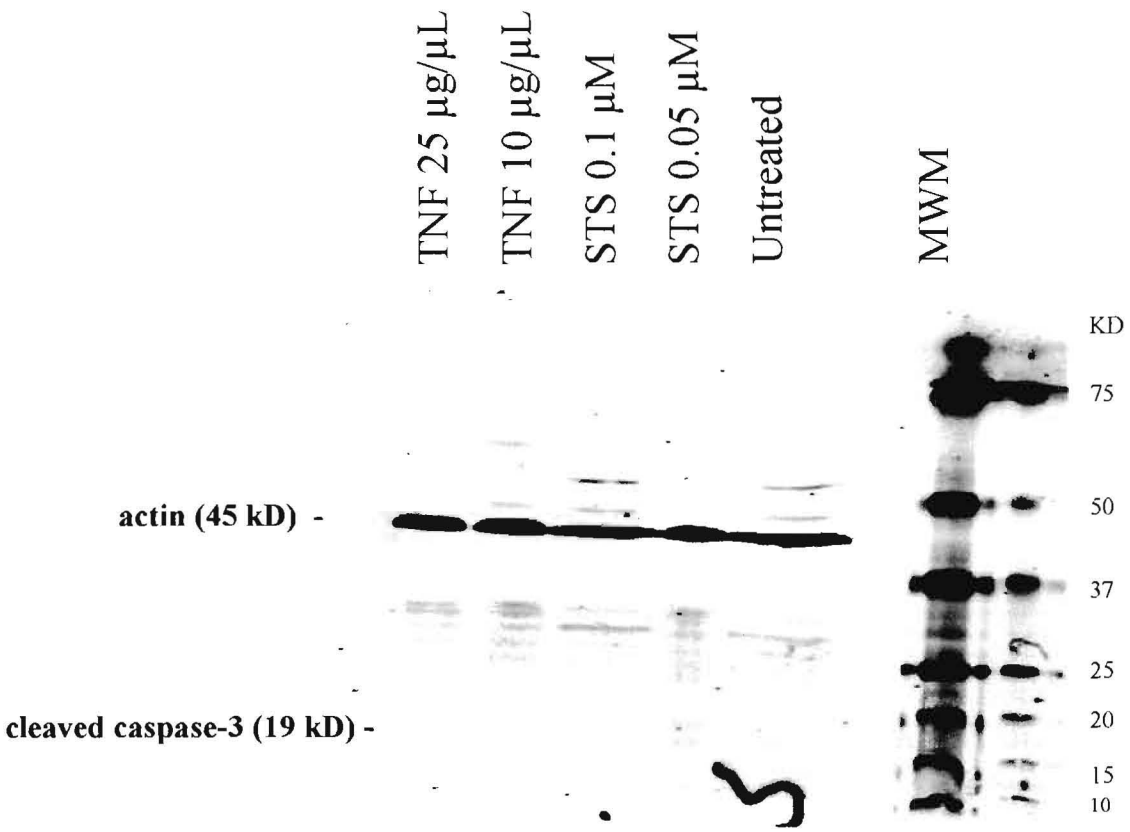

Figure 2. Western blot of Jurkat cell extracts probed with actin and cleaved caspase-3. Jurkat cells were either untreated or treated for 24 hrs with STS (0.05 µM or 0.1 µM) or TNF (10 µg/µL or 25 µg/µL). The Odyssey Imaging System was used to determine band intensity and to correct contrast issues caused by the background.

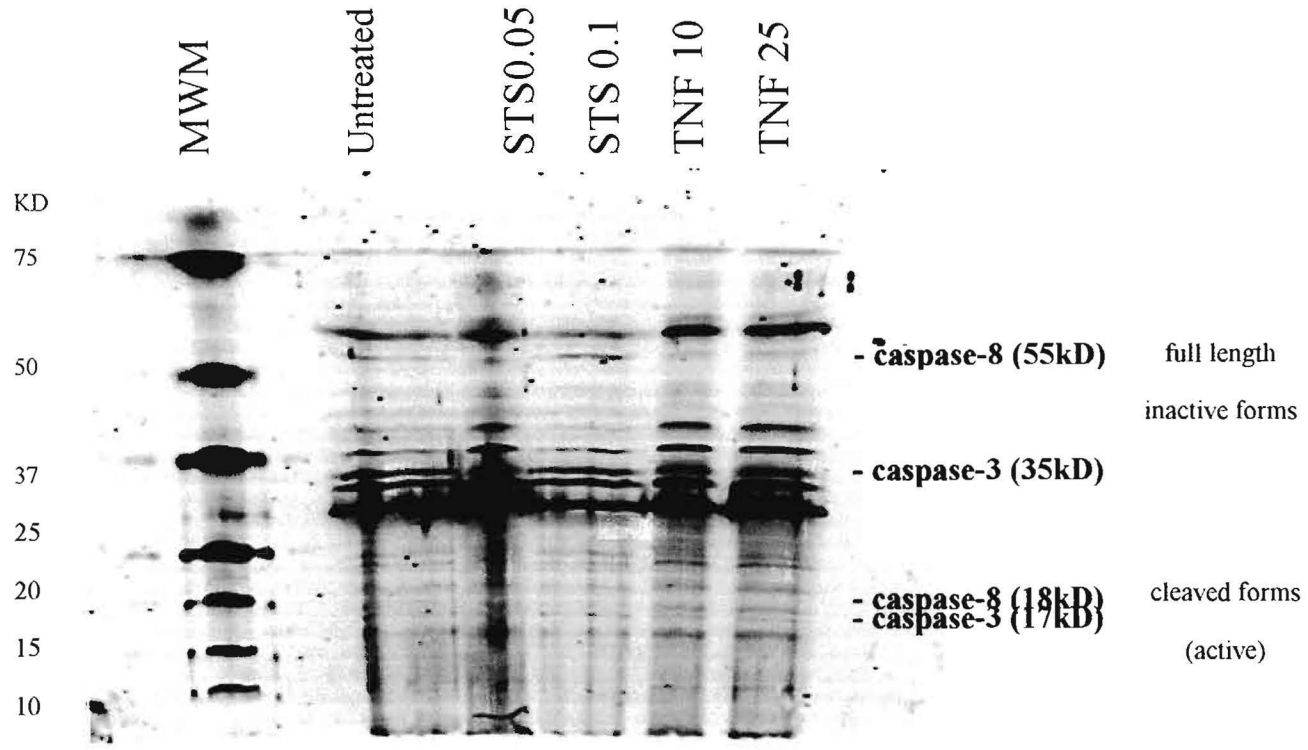

Figure 3. Western blot of Jurkat cell extracts probed with caspase-3 and caspase-8. Jurkat cells were either untreated or treated for 24 hrs with STS (0.05 μM or 0.1 μM) or TNF (10 μg/μL or 25 μg/μL). The Odyssey Imaging System was used to determine band intensity and to correct contrast issues caused by the background.

Caspase-3 Activity

Figure 5 depicts the results of caspase-3 activity from all cell extracts. Duplicate readings of caspase activity were generated in a 96-well plate and read at 405 nm absorbance. The absorbance value of each sample represents the amount of caspase-3 activity present. This absorbance was then subtracted from the blank to generate the value needed for creating a bar graph to compare all the extracts. Overall, absorbance values measured were relatively low, but

there were differences across all treated samples. Activity levels were highest in the STS 0.05 μM and TNF 25 μg/μL extracts (Figure 5). The calculated value for untreated extracts was 0.0203. This value was anticipated to be low as it contained untreated cells with minimal caspase-3 activity. The calculated absorbance value for STS 0.05 was 0.1149. The caspase-3 activity increased 0.0946 absorbance units, which showed that caspase-3 activity was present. The calculated absorbance value for STS 0.1 was 0.0358. The caspase-3 activity increased 0.0155 absorbance units, which followed the same trend that treated cells had higher caspase-3 activity than untreated cell extracts. The TNF 10 sample had an absorbance value of 0.0639, an increase of 0.0436 units in activity. In the TNF 25 sample, there was an absorbance value of 0.1288 calculated that created a unit increase of 0.1085 in caspase-3 activity. In TNF samples, values calculated showed that caspase-3 activity did increase with higher drug concentrations.

In this caspase-3 activity assessment, TNF 25 induced the most caspase-3 activity. The second highest activity was seen in the STS 0.5 extract. This information confirms the results from the cell count data as STS 0.5 and TNF 25 were shown to have the most cell death. In terms of the correspondence between caspase-3 activity and Western blotting, the data was harder to interpret. In Figure 3, the expression of caspases reflected STS 0.5 and TNF 25 inducing the most caspase activity. However, the other figures did not show expression of caspases at a level detectable through Western blotting. Figures 1 and 2 show minimal expression of caspase activity and Figure 4 shows the most expression in STS 0.5 and TNF 10. These results indicate that cell counts, Figure 3 and the caspase-3 activity assay confirm STS 0.5 and TNF 25 generating the greatest caspase activity and expression.

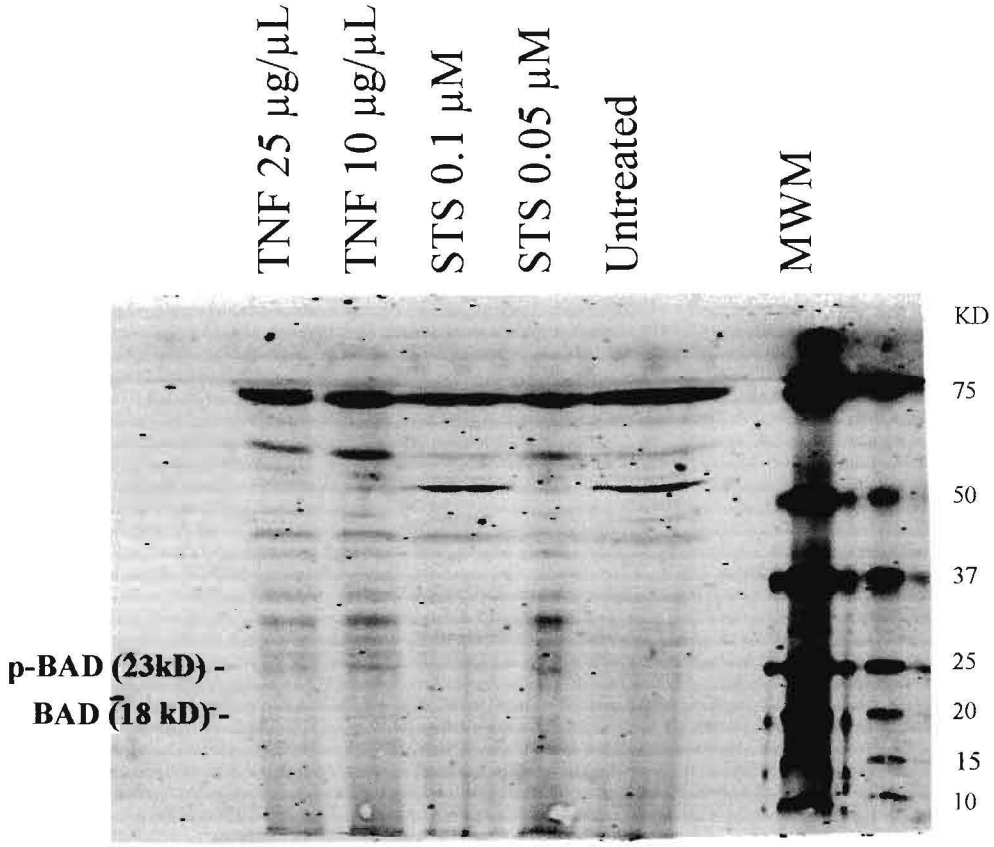

Figure 4. Western blot of Jurkat cell extracts probed with BAD and p-BAD. Jurkat cells were either untreated or treated for 24 hrs with STS (0.05 µM or 0.1 µM) or TNF (10 µg/µL or 25 µg/µL). The Odyssey Imaging System was used to determine band intensity and to correct contrast issues caused by the background.

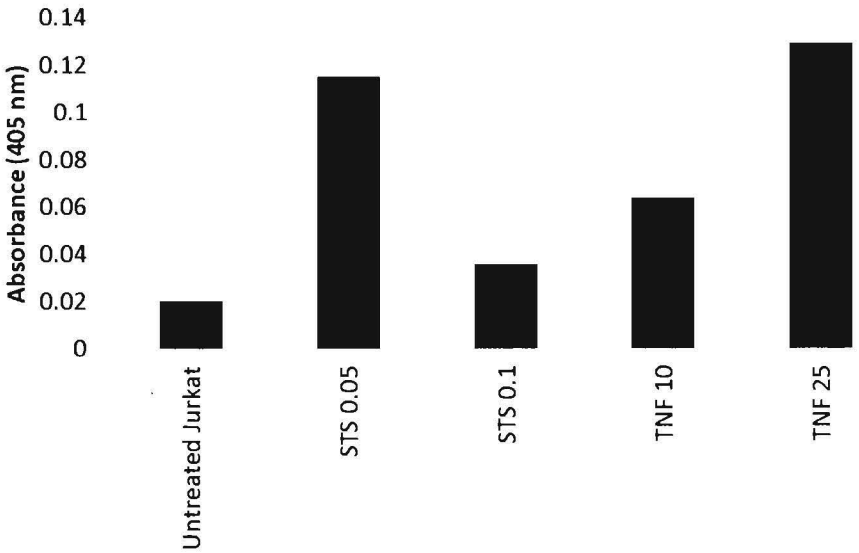

Figure 5. Measurement of caspase-3 activity in Jurkat cell extracts. Samples were incubated for 4 hrs at 37°C. Absorbance values were read at 405 nm for each sample. Activity values obtained were calculated using formulas provided in the Promega caspase-3 activity protocol to generate mean absorbance for each sample.

Discussion

This research focused on changes in protein expression to determine if drug treatments had an effect on apoptosis observed in both treated and untreated Jurkat cells. Through cell counts, it was determined that cells were indeed dying through 24 hr treatments and there were differences in the amount of cell death based on drug concentrations. Drug treatments were used to determine if lower drug concentrations would be able to induce apoptosis comparable to results seen with higher drug concentrations. Both cleaved and uncleaved caspase-3 and caspase-8 were assessed to determine if these caspases were active and were contributing to the overall cell death results. Western blot data showed that when Jurkat cells were treated with STS, there was generally less expression of caspases than that of TNF-treated cells. This was also confirmed by the data generated through initial cell counts. In every cell count conducted, TNF-treated cells exhibited more cell death than STS-treated cells (Table 1). Each TNF treatment showed a decrease in cells, with TNF 25 inducing the most apoptosis. STS treatments also displayed a decrease in cells, although the results were not consistent through all experiments conducted. All of the drug treatments showed a decrease in cells from the untreated control, but two of the STS 0.1 cell counts resulted in an increase in cells. Both TNF extracts always showed a decrease in cells from the initial untreated Jurkat cell count. Based on the four Western blots, band intensity of any protein probed for in STS 0.5 and STS 0.1 treatments was either equal to or less than that of TNF treatment. In this research, it was hoped that the drugs would have similar effects on inducing apoptosis in cells as basic staurosporine research states that STS targets all cells that contain caspases (22). All living cells have a standard amount of caspases, cleaved and uncleaved, so, staurosporine has the opportunity to enter cells and induce apoptosis without additional cell signaling (20).

Yet, even with staurosporine's caspase affinity, TNF treatment was more effective in increasing caspase activity to promote apoptosis in this set of experiments. TNF was able to induce the most apoptosis because of its ability to activate ICE (Interleukin 1 β-converting enzyme) and CED-3 (cell death defective), cysteine proteases (21). Ectopic or overt expression of these proteases has been shown to induce apoptosis. As evidenced by the dark bands, TNF-treated Jurkat cells showed greater intensity of cleaved caspase-8, uncleaved caspase-3 and uncleaved caspase-8, in almost all treatments made (Figure 4). From known caspase-3 information, inactive caspase-3 becomes cleaved into the active form by caspase-8 and caspase-9. So, it was very important to the research that there be sufficient amounts of full-length inactive caspase-3 to be cleaved by caspase-8 and activate the apoptotic cascade. Based on the intensity of bands seen in Figure 4, caspase-3 was present in abundance to carry out apoptosis through caspase cascades in all treated extracts.

Thus far, results observed suggest that TNF-treated cells showed higher amounts of active caspase-3 and caspase-8 activity than untreated Jurkat cells or STS-treated cells. In this research, it was apparent that the TNF extracts had more induced apoptosis than the STS extracts. These treatments used varying concentrations of drugs and, it seemed, that TNF through its receptor-mediated nature led cells to apoptosis more effectively than STS through its mitochondrial-mediated pathway. And, to ensure credibility of the data generated, actin antibody was used as a loading control. As was evidenced again and again, all the samples showed equal loading in all lanes and this helped to reinforce the fact that band intensity was not impacted by the amount of protein loaded.

From the perspective of caspase activity, the research was based around the knowledge that caspase-8 and caspase-3 are part of the receptor-mediated and mitochondrial-based apoptotic

pathways. As referenced before, apoptosis is a regulated cellular process needed to support normal cell growth and function through the action of caspases. Here, caspase-8 initiates the signal that in turn activates caspase-3 and begins the cascade of caspase activity (19). We were most interested in the cleaved forms of caspase-3 and caspase-8, as these forms are responsible for the apoptotic cascade that takes place in the cell. In general, caspase activity was higher in treated Jurkat cells than untreated Jurkat cells. By comparing the caspase-3 Western blot to the caspase-3 activity data, both effectively showed untreated cells had the lowest expression of caspase-3. The Western blot for caspase-3 also showed moderate expression of the caspase. Untreated Jurkat cells did exhibit minimal caspase-3 activity, which is to be expected. However, as cell life and death are programmed and heavily regulated within the cell, caspase-3 activity is not expected to vary outside of a certain range to maintain the stability of cells.

Some variances in loading were observed in the STS 0.1 μM treated cell extracts. Caspase-3 activity of the STS 0.1 sample should have been similar to that of the TNF 25 sample. If not the same in intensity, the higher drug concentration of STS should have induced more cell death than the lower drug concentration. As both the STS and TNF treatments were completed at a low and high concentration, the higher concentration of STS should have induced greater caspase-3 activity similar to that of the TNF treatment. However, the STS 0.1 caspase activity was lower than that of the STS 0.05 sample, which should not be occurring. This may be due to issues with protein extraction from the 0.1 μM STS cells, which did prove problematic in the earlier protein determination of the sample. Also, there were loading differences between the untreated Jurkat cells, STS 0.1 μM cells and the other extracts. This may have caused some of the variances in band intensity due to unequal loading. However, as stated in the Results section,

actin expression confirmed equal loading in all samples. Thus, it can be assumed that the loading error was due to something such as incorrect protein extraction or determination.

Based on the results, apoptosis was induced in each treatment of cells as evidenced by the protein expression in each treated sample. Also, when samples were probed for Bad and p-Bad, higher caspase expression was observed which lends credence to the fact that apoptosis occurred. As Bad is part of the BCL family responsible for binding to pro-survival family members, expression of this protein would indicate that apoptosis signaling might be occurring within the extracts. Since Bad expression was moderate in both TNF extracts and the STS 0.05 extract, it can be suggested that apoptotic pathways were being carried out in these extracts. This was validated by the lack of p-Bad expression in all extracts. P-Bad acts to bind Bad and initiate pro-survival pathways in cells (18). Because p-Bad was not expressed, it can be inferred that apoptosis was allowed to take place and that all extracts were signaled to initiate cell death.

Through the course of this research, it has become apparent that consistency in methods of preparation of extracted protein has a direct impact on the results seen in Western blots and in caspase activity assays. These results have shown that even slight variations could lead to differences in loading and overall expression of proteins in a given sample. Although the untreated extract displayed the same irregular loading as the higher concentration of STS, it did not exhibit the lowered intensity of caspase-3, caspase-8 and p-Bad/Bad expression throughout the Western blots like the 0.1 STS treatment did. Because of these irregularities, it is believed that incorrect protein determination may have been the determining factor in properly assessing protein expression of both caspases in the STS 0.1 extract. In the case of the untreated extracts, the amount of caspase-3 and caspase-8 expression remained minimal, which supports the assumption that caspase activity occurs regularly within cells. As for the STS 0.1 sample,

expression of capase-3 and caspase-8 was lower than normal and did not show what was expected. If the STS- and TNF-treated extracts were compared, STS treatment should follow roughly the same pattern where the STS 0.1 extract should have had greater expression of caspases than that of the STS 0.05 treated extract. The Western data of lowered active and inactive caspase activity in the STS 0.1 extract was supported by the caspase-3 activity results. Higher concentrations of drug should result in increased caspase activity, which then leads to apoptosis. As this was not the case in the STS treatment, further research would need to be done to ensure full extraction of proteins occurred before performing experimental assays. In this respect, values recorded and observed may have been influenced by protein extraction issues yet again. It is hoped that, in future research, even more precise adherence to protocols for protein extraction, determination and Western blot image generation will lead to clearer, more concise data supporting the direct correlation between caspase expression and apoptosis in Jurkat cells.

The results of this research suggest that active caspase-3 and caspase-8 protein expression is directly correlated to the amount of cell death that occurs within the Jurkat cell line. The cleaved forms of these caspases are able to more efficiently lead to apoptosis through receptor-mediated pathways, of which TNF was the most reliable in terms of continued caspase activity. It is believed that TNF had the greater advantage in this research because of its heavy use of receptors to promote apoptosis. STS is a non-specific protein kinase c inhibitor, which causes apoptosis through increasing the intracellular accumulation of cytotoxic elements (23).

In this research, caspase-3 exhibited the highest expression, even although it is known that caspase-8 is required to activate caspase-3. Hence, caspase-8 is only needed in moderation to initiate the cleavage of caspase-3 leading to the cleavage of other proteins. Figure 3 displays that caspase-8 was present in both the active and inactive forms. It also showed the most intense band

was observed for the full-length caspase-3. As both caspase-3 and caspase-8 had increased expression, apoptosis had occurred. In light of this information, it was still determined that both of the drugs and the varying concentrations used did induce higher caspase activity than that of the untreated extracts. However, more specific testing such as more rigorous caspase-3 and caspase-8 fluorescence activity assays, ApoTox-Glo assays (determines the mechanism of cell death by drug) and flow cytometry must be done in order to fully understand the best combination of drugs to produce effective apoptosis. It is hoped that the data generated in this research will spark interest in the individual roles of caspase-3 and caspase-8 and the relationship between their activation and the overall apoptotic response induced by TNF and STS.

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